



Soluble adenylyl cyclase in health and disease[☆]

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ABSTRACT

The second messenger cAMP is integral for many physiological processes. Soluble adenylyl cyclase (sAC) was recently identified as a widely expressed intracellular source of cAMP in mammalian cells. sAC is evolutionary, structurally, and biochemically distinct from the G-protein-responsive transmembranous adenylyl cyclases (tmAC). The structure of the catalytic unit of sAC is similar to tmAC, but sAC does not contain transmembranous domains, allowing localizations independent of the membranous compartment. sAC activity is stimulated by HCO_3^- , Ca^{2+} and is sensitive to physiologically relevant ATP fluctuations. sAC functions as a physiological sensor for carbon dioxide and bicarbonate, and therefore indirectly for pH. Here we review the physiological role of sAC in different human tissues with a major focus on the lung. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease, guest edited by J. Buck and L.R. Levin.

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1. Introduction

The second messenger cyclic adenosine monophosphate (cAMP) was discovered in 1958 [1,2]. Since then, it is recognized as one of the most important and evolutionarily conserved second messengers for many signaling pathways [3]. cAMP can activate three main effector proteins: cyclic-nucleotide-gated (CNG) ion channels [4], guanine-nucleotide exchange proteins activated by cAMP (Epac) [5] and cAMP-dependent protein kinase (PKA) [6,7].

cAMP is generated from ATP by adenylyl cyclases (ACs). There are six different classes of ACs distributed throughout bacteria, archaea and eukarya. These classes are unrelated in sequence and structure, but all produce cAMP [8]. All eukaryotic adenylyl cyclases belong to class III [3]. Vertebrate animals were felt to have only one family of hormone and G-protein regulated enzymes with a transmembranous component (tmAC). In mammals, this family contains nine tmACs transcribed from 9 different genes, which differ in their tissue and developmental expression as well as in their regulatory properties [3,9]. In 1999, Levin and Buck cloned a genetically unrelated AC in rat testis, guided by a cyclase activity originally described in the 1970s that was different from tmAC [10,11]. The activity was originally described by Braun in 1975 as a Mn^{2+} responsive AC in rat testis [12]. At discovery, the enzyme was named “soluble Adenylyl Cyclase” (sAC) as it was found

in the cytosolic compartment of rat testis preparations [12]. Later, it was shown that most of sAC was not soluble in the cytoplasm, but found in discrete locations such as the nucleus, mitochondria, centrioles or cilia [13–15]. Fig. 1 summarizes the activation mechanisms of different adenylyl cyclases.

2. Structure of sAC

Mammalian nucleotidyl cyclases contain two fairly well preserved catalytic domains. These two domains (C_1 and C_2), by associating with each other, form the catalytic core. The C_1C_2 heterodimers shape into two sites at the interface: the active site and a degenerated, inactive pocket [16,17]. sAC and tmACs are monomeric proteins and catalyze cAMP production through dimerization of their two catalytic domains [18]. They share homology of the two catalytic domains, but sAC lacks 2 hydrophobic domains, each representing 6 membrane-spanning helices that localize tmAC to membranes [19].

Recently, the crystal structure of the catalytic domains of sAC was described [17,20]. The human catalytic units reveal a secondary structure similar to the one from cyanobacteria but differences are seen in some external loops. The cyanobacterial sAC has two fully identical nucleotide binding sites. In contrast, only one of these sites is accessible for ATP in the human form [20]. Interestingly, there is a sequence of 3 consecutive proline residues between C_1 and C_2 (220–222) locally related to a hydrophobic patch [17]. These structures have been described as potential protein binding sites [21]. They offer the possibility of an interaction area for proteins or other sAC domains [17] which could allow dimerization of sAC splicing forms that only contain one catalytic unit.

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In contrast to other mammals as the dog or some anthropoids, humans have a single sAC gene [22]. By alternative splicing, several sAC isoforms are generated [15,23,24] and an additional alternative start site has recently been described, indicating a considerable isoform diversity [25]. Full-length sAC (sAC_{fl}) includes an N-terminus with the two catalytic domains (~1100 amino acids spanning 33 exons). Exclusion of exon 12 generates a truncated isoform, sAC_t (amino acids 1–490), which contains just the two sAC catalytic domains [17]. Although the half maximal stimulation for HCO₃⁻ and the K_m for Mg²⁺ and Mn²⁺ and Ca²⁺ are the same for sAC_t and sAC_{fl}, the truncated form is 10 times more active than sAC_{fl} [10,26]. This is explained by an autoinhibitory domain in the C-terminal tail of sAC_{fl} [27] that is not present in the truncated form. Splice variants of the sAC_{fl} found in testis and skeletal muscle also contain a heme-binding domain that could bind NO or CO. A detailed description of alternative splicing in bronchial epithelial cells will be given below.

3. Cell compartmentalization and microdomains

cAMP is a second messenger that can signal at different locations in a single cell [7]. For this purpose, a tight spatial and temporal control of the cAMP concentration is critical. The cell has two strategies to do this. First, cAMP production and utilization are spatially confined. Whereas tmACs are restricted to membranes, sAC can be localized throughout the cell and is found in mitochondria, nuclei, centrioles, the mitotic spindle [13] as well as cilia [15,28]. At these locations, ACs are anchored together with PKA [29,30] by scaffold proteins such as A-kinase anchoring proteins (AKAP), allowing local utilization. Second, the diffusion of cAMP is confined by the degradation of cAMP by PDEs

[31] and the apical actin web, which requires functioning cystic fibrosis transmembrane conductance regulator (CFTR) in some epithelial cell types [32]. This fence mechanism together with the local production and utilization of cAMP creates “microdomains” of cAMP signaling.

Microdomains of cAMP signaling may not be captured by whole cell cAMP measurements. Better suited are measurements in intact cells using reporter protein constructs (e.g., PKA or Epac) that allow recordings in subcellular compartments [33], for instance, using fluorescence resonance energy transfer (FRET).

4. Regulation of sAC

The product of both sAC and tmAC is cAMP, but the regulation of the two enzymes is completely different. G-protein-coupled receptors and heterotrimeric G proteins [34] regulate tmAC, allowing a broad spectrum of signaling input. These regulation mechanisms do not apply to sAC. Forskolin, a plant diterpene that binds to the degenerated pocket of the catalytic unit of tmACs [17], triggering a strong enzymatic response, does not affect sAC signaling. An inserted loop in the three dimensional configuration of sAC's catalytic domains tightens the available space in the degenerated pocket, preventing forskolin binding [17].

sAC is directly activated by HCO₃⁻, leading to increased substrate turnover and reduced substrate inhibition and by Ca²⁺, enhancing substrate binding [35]. In this way, small changes in the intracellular concentration of bicarbonate and calcium ions may significantly affect local cAMP levels [36]. Finally, sAC is sensitive to variations in intracellular ATP concentrations [37]. These regulation mechanisms have been preserved throughout evolution from cyanobacteria to humans [17], supporting the importance of sAC in cell signaling.

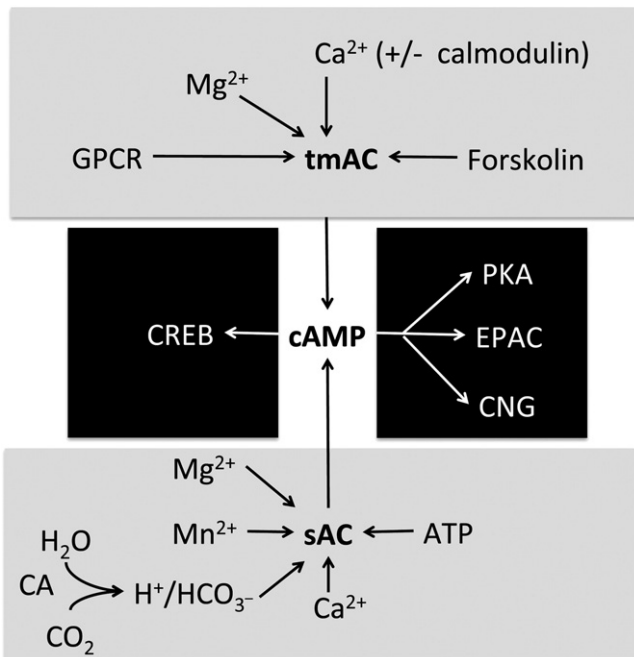


Fig. 1. Regulation of cAMP production. Upper Panel: cAMP is produced by tmAC, which can be stimulated by G-protein coupled receptors (GPCR). tmACs are also stimulated by forskolin, a diterpene that is not activating sAC. tmACs require bivalent ions such as Mg²⁺ and Ca²⁺ for the cyclization of cAMP from ATP. Calcium signals activate some of the 9 known tmAC over calmodulin whereas calmodulin is not required for signaling in some other tmACs. Lower Panel: sAC is stimulated by HCO₃⁻ which is in an equilibrium with H₂O and CO₂ catalyzed by carbonic anhydrase (CA). sAC also requires calcium (independent of calmodulin) for cAMP production and is stimulated by magnesium. Magnesium can be substituted by manganese. Middle Panel: cAMP signals over different mechanism in the body: Protein kinase A (PKA), the guanine-nucleotide exchange proteins activated by cAMP (Epac) and the cyclic-nucleotide-gated (CNG) ion channels. In the nuclear compartment cAMP can affect gene expression over cAMP response element-binding protein (CREB).

4.1. Calcium

Calcium plays a role in regulating sAC and certain tmACs. At physiological concentrations, Ca²⁺ stimulates the tmACs AC1, AC3 and AC8 isoforms via calmodulin and inhibits AC5 and AC6 by displacing the co-factor Mg²⁺ [36,38]. In contrast, mammalian sAC is stimulated by Ca²⁺ in a calmodulin independent way [3,26,35] by lowering the K_m for Mg²⁺ ATP [20]. Like other class III adenylyl cyclases, sAC requires two divalent cations for activity. It is active if Mg²⁺ is the only available divalent ion, but addition of Ca²⁺ increases the affinity for its substrate ATP to values consistent with the concentration of ATP found inside the cells [35]. This suggests that in vivo, mammalian sAC utilizes both Mg²⁺ and Ca²⁺, and that its activity will be sensitive to ATP fluctuations inside the cells. Modeling of sAC activity suggests that Ca²⁺ bound to the γ-phosphate of ATP enters the catalytic site where it interacts with specific residues in the sAC catalytic center, resulting in an “open sAC state”. Mg²⁺, the second divalent metal then binds to the α-phosphate of ATP, leading to a “closed state”. The change from “open” to “closed” states induces the release of the β- and γ-phosphates and esterification of the remaining α-phosphate with C3 of the ribose in adenosine (“cyclizing”) [19].

4.2. Bicarbonate

HCO₃⁻ stimulates substrate turnover via an allosteric change of sAC leading to closure of the active site, recruitment of the catalytic Mg²⁺, and rearrangement of the phosphates in the bound ATP as it has been shown in the sAC homolog of cyanobacterial adenylyl cyclase (CyaC). This facilitates cAMP formation and release [19].

Through its HCO₃⁻ regulation, sAC has been shown to function as a physiological CO₂/HCO₃⁻ (and thus indirectly pH) sensor in many tissues [39]. The sAC-dependent mechanisms for sensing HCO₃⁻ in extracellular fluids are similar in shark gills, kidney and epididymis using either HCO₃⁻ movement through channels or diffusion of CO₂ through the membrane [40,41]. This becomes particularly effective when sAC

is associated with carbonic anhydrases that regulate the HCO_3^- concentrations [3]. Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of CO_2 to H_2CO_3 . There are 10 enzymatically active members in the CA gene family. CAs I, II, III and VII are cytoplasmic whereas CAs IV, IX, XII and XIV are membrane bound [42]. CAs II and IV have been found in the canine airway epithelium, at different locations between the larynx and the alveolar compartment. While CA II is fairly evenly distributed in all compartments, CA IV expression is high in the laryngeal area and also prominent in the intrathoracic airways, mostly in the area of the carina and the lobar bronchi [43].

The EC_{50} for HCO_3^- of sAC in vitro is close to the normal serum concentration of HCO_3^- , about 20 mM in mammals, implying that small changes in HCO_3^- will lead to large changes in sAC activity around this concentration [11,35]. Intracellular HCO_3^- in mammals is between 10 and 15 mM [44]. When considering the organization of cAMP signaling in microdomains with sAC, phosphodiesterases as well as CAs, HCO_3^- can be rapidly changed either by increased CO_2 production or CO_2 influx from the interstitium. This mechanism is believed to play an important role in ciliary beat frequency (CBF) regulation as discussed below.

4.3. sAC inhibitors

Several inhibitors are commonly used to inhibit sAC. KH7 and 2',5'-dideoxyadenosine (ddAdo) help identifying the source of cAMP in cells as KH7 effectively inhibits sAC-dependent cAMP accumulation without affecting tmACs, whereas ddAdo blocks the p-site of tmAC without affecting sAC [39]. Another potent p-site inhibitor of tmAC is ddATP, but it can only be used in cell lysates as it does not permeate the cell membrane [39]. The mechanism of these different binding behaviors is still not completely understood [39]. While KH7 is the most specific sAC inhibitor [39], it still has non-specific effects [45,46]. Based on its intrinsic fluorescence, its use in fluorescent microscopy has to be carefully planned and well controlled [39]. As additional inhibitors for sAC, 2- and 4-hydroxycatechol estrogens (2-CE and 4-CE) have been described [39, 47]. Interestingly and for unknown reasons, these inhibitors act on tmAC and sAC in vitro, but only on sAC in cell-based systems [39].

4.4. sAC regulation patterns

sAC can regulate biological processes using at least two different patterns: as a modulator of an underlying baseline amplitude or as an on-off mechanism. The modulator function of sAC has been found in the regulation of oxidative phosphorylation in mitochondria [48] where sAC alters ATP production dependent on CO_2 production. A similar effect can be seen in ciliary beat frequency (CBF) regulation in the airways [15] as described below. A typical example for an on-off mechanism is the capacitation of sperm for egg fertilization [49]. Sperm also uses sAC to modulate their flagellar motility in a modulatory fashion.

5. Role of sAC in health and disease

CO_2 , HCO_3^- , and pH/H^+ are inseparably linked in biological systems. Due to CAs, CO_2 is nearly instantaneous in equilibrium with H_2CO_3 , which in turn rapidly dissociates into H^+ and HCO_3^- . Changes to any one of these molecules are reflected by variations in the other two, and all three (CO_2 , HCO_3^- and pH) play central roles in biology [22].

While all cells possess mechanisms to sense and respond to changes in CO_2 , HCO_3^- , and H^+ , multicellular organisms have specialized cells which measure intracellular HCO_3^- and pH_i as surrogates for the levels of CO_2 , pH and HCO_3^- in their immediate environment [22]. These cells can respond to stimuli by secretion/absorption of H^+ , HCO_3^- or other ions, regulating the ventilatory rate to control CO_2 exhalation and change metabolism and regulation of gene expression [22].

sAC has been first detected in testes and spermatozoa. Its role was initially thought to be confined to fertility and capacitation [50,51],

supported by the finding that sAC knockout mice were viable but infertile [25]. In recent years, however, the recognition of somatic sAC functions has been exploding. We will therefore provide an overview of known sAC signaling with a major focus on the lung.

5.1. sAC in the lung

In cultures of human airway epithelial cells, we initially found 3 splice variants of sAC [15]. One variant had two catalytic domains in the N-terminal region, whereas the two other versions initiated translation from a new ATG codon that is in frame with the C-terminal open reading frame of the longest version. This leads to a smaller protein, missing the first 97 amino acids, cutting out a part of the first catalytic unit but leaving the second functional [15].

A recent detailed analysis of sAC splice variants in human airway epithelial cells [28] identified full-length sAC and many C_2 only sAC variants. The C_2 only sAC transcripts could be divided into two groups. Group 1 transcripts initiated at the original start codon with C_1 disruptions or deletions (i.e., skipping exon 5 or exons 3 and 5), while Group 2 transcripts initiated translation from an alternative start codon. Many of these partially retained nucleotides from the 3' end of previously annotated intron 4 inserting an in frame termination codon and a new translation start codon. Given expression data and reported western blots, sequences of these group 2 variants are expressed and the new exon 5 was therefore labeled 5v2. Except for full-length sAC, all alternatively spliced variants have a complete C_2 but an incomplete C_1 . This is similar to the mouse somatic sAC isoform found in *Sacy^{tm1Lex}/Sacy^{tm1Lex}* 'knockout' mice, which also only contains the C_2 only variant except that transcription of this mRNA initiates from a new promoter upstream of exon 5 and starts translation from an ATG codon in exon 6 [25].

5.1.1. Mucociliary clearance

The airways span from the nose to the respiratory bronchioles representing the portal to the alveolar space. Besides conducting air to the alveoli, the airways are responsible for cleaning the air from all kinds of contaminants, such as dust and microorganisms. The major mechanism for this process is mucociliary clearance (MCC), which is dysfunctional in multiple chronic airway diseases such as chronic bronchitis and cystic fibrosis. To accomplish efficient clearance, three crucial components must work together: proper ciliary beating, mucus hydration and composition as well as airway surface liquid availability. The airways are covered with a carpet of cilia that provides the motor to propel material impacting on the apical mucus out of the lungs. Mucus-producing cells are embedded between ciliated cells in the superficial epithelium and in submucosal glands. Mucus serves as a trap for the inhaled particles. It is floating atop cilia that are surrounded by periciliary fluid, a low viscosity layer containing glycoproteins and other substances [52]. In this layer, cilia beat with their effective beat from distal to proximal to propel mucus out of the airways. Cl^- , Na^+ and K^+ channels in the apical (and K^+ channels in the basolateral) membrane of airway epithelial cells regulate the height of the periciliary layer. cAMP signaling is critical for the regulation of some of these channels as well as for the regulation of CBF.

5.1.2. Cilia and ciliary beat frequency

Cilia beat in a coordinated fashion, creating metachronal waves [53]. Hydrodynamic forces passively regulate ciliary coordination and metachronal waves. Thus, the regulation of cilia themselves becomes crucial. Baseline ciliary beat frequency (CBF) and changes in CBF are modulated by cAMP, intracellular pH , Ca^{2+} and HCO_3^- .

The effect of cAMP on the axoneme is stimulatory in mammals. It is mediated by ciliary PKA, which phosphorylates an outer arm dynein light chain [54]. The phosphorylation of this light chain is sufficient to speed up microtubular sliding and CBF in paramecium. The role of sAC in this process remains only incompletely solved. The cell has multiple

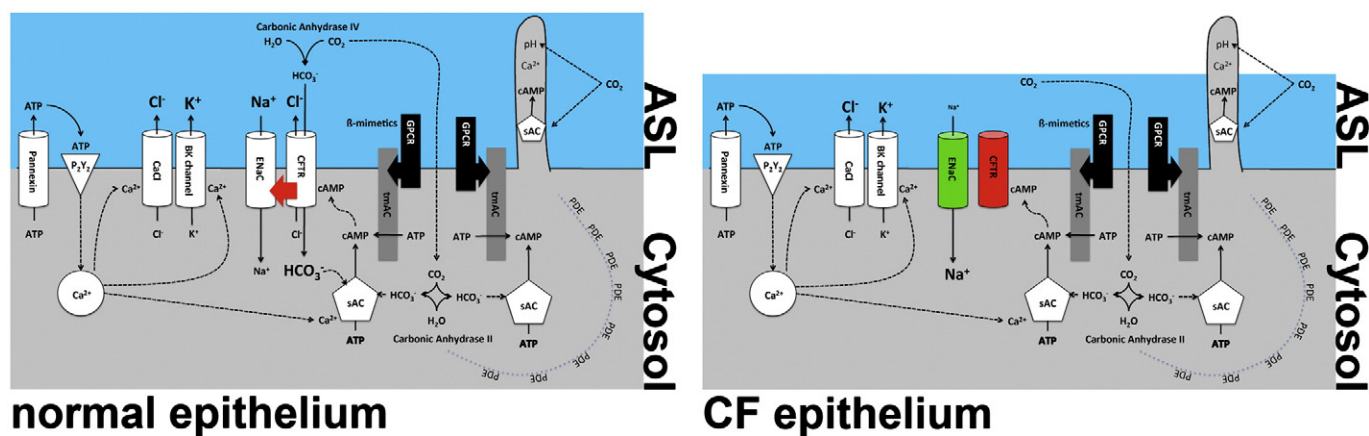


Fig. 2. Airway surface liquid (ASL) regulation in normal and CF epithelium. Left panel: ASL is mainly regulated via chloride excretion and sodium absorption. The major chloride excretion occurs via CFTR, which is stimulated by cAMP that can arise from tmAC as well as from sAC. CFTR is also conducting HCO_3^- that can stimulate sAC and builds a self-enhancing mechanism. The second chloride channel is dependent on calcium and functions together with potassium excretion via BK channels, which is also dependent on calcium. Sodium is absorbed via the epithelial sodium channel (ENaC) that is inhibited by CFTR. sAC was also found in cilia, where it increases ciliary beat frequency (CBF) over the production of cAMP, an effect dependent on intracellular HCO_3^- concentrations. cAMP is locally confined by the presence of phosphodiesterases (PDE) and the actin cytoskeleton. Right panel: In patients with CF, the major chloride transport channel (CFTR) is not working, which also leads to a missing inhibition of ENaC. Together, these changes lead to a decreased height of the ASL, which is felt to be the major cause of the pulmonary disease of CF patients. Additionally, HCO_3^- cannot get into the cells and cannot stimulate ciliary beating, which further decreases mucociliary clearance.

possibilities to increase the cAMP over G-protein coupled receptors, such as β_2 adrenergic [55] and adenosine 2b receptors [56], but it is unclear whether cAMP produced in the cytosol can diffuse into cilia in relevant concentrations. The exact mechanisms of how G-protein coupled receptors increase beating frequency remains incompletely understood as tmACs were not found in ciliary membranes [57]. We speculate that sAC must play a role as it is the only known intraciliary source of cAMP [28].

Cells can also increase Ca^{2+} and stimulate calcium-sensitive tmACs [57] or sAC to increase cAMP. Elevation of $[\text{Ca}^{2+}]_i$ is associated with an increase of CBF in mammals. The changes in CBF initiated by Ca^{2+} require only small elevations of Ca^{2+} and occur within one beat cycle [58,59]. The mechanism of Ca^{2+} -mediated changes of CBF is not fully understood, but seems at least in part to be a consequence of a direct Ca^{2+} action on a ciliary target [58]. On the other hand, it has also been shown that increased Ca^{2+} can have an effect on CBF via cAMP, likely through sAC [60].

CBF is reacting to intracellular pH changes [61], whereas extracellular pH barely affects the frequency [62,63]. This effect is independent of protein kinases and phosphatases [61] and supports a direct effect of pH on the axoneme.

Finally, CBF is modulated by HCO_3^- in a pH-independent way via production of cAMP by sAC [15,64]. It has been shown that the HCO_3^- related increase in CBF can be blocked by the sAC inhibitors KH7 and 2-CE and PKA inhibition, but not by inhibition of tmAC [15].

Given the splice variants in airway epithelial cells described above, we wanted to know which one of these was targeted to cilia. One of the splice variants, spanning the two new exons 5v2 and 12v2, encodes a ~45 kDa protein which is about the size of the ciliary variant detected in cilia by western blotting [15,28]. When this variant was expressed in human and murine airway epithelial cells, it was indeed found in cilia. In vitro, a discovered cytoplasmic variant containing both catalytic domains (similar to truncated sAC, ~55 kDa) was producing cAMP but the ciliary variant was not. While C_2 sAC KO mice lost their sAC-dependent CBF control, recombinant expression of the ciliary sAC variant rescued CBF regulation in a sAC-dependent manner, suggesting that this variant was active in vivo. On the other hand, expression of the cytoplasmic variant did not rescue CBF, indicating that cAMP produced by this splice, which was active in vitro, could not freely diffuse into cilia. In summary, analysis of sAC variants revealed for the first time a mammalian axonemal targeting sequence. The ciliary variant indeed rescued sAC-dependent CBF regulation in C_2 KO mice despite

having only a complete C_2 domain, suggesting that this variant recruits a C_1 domain donor for activity [28].

5.1.3. Regulation of airway surface liquid and the periciliary fluid

The airway epithelium uses apical ion transport to regulate the volume and composition of the airway surface liquid (ASL) to optimize MCC [65]. In the airways, luminal chloride secretion via cystic fibrosis transmembrane conductance regulator (CFTR) and other Cl^- channels as well as a balance of Na^+ absorption is essential for maintaining adequate ASL volume. Current evidence suggests that changes in ASL volume and composition are essential in the pathogenesis of cystic fibrosis lung disease [66]. CFTR is regulated by cAMP, partially related to sAC [67]. Besides chloride, CFTR may conduct (or absorb) HCO_3^- along a concentration gradient in some epithelial cell types. There are also Ca^{2+} dependent chloride channels in the apical membrane (CaCC). They are stimulated via Ca^{2+} release after purinergic stimulation of P_2Y_2 receptors. The driving force of this chloride outflow is provided by a Ca^{2+} activated and voltage-dependent potassium (BK) channels [68].

5.1.4. Cystic fibrosis and sAC

Cystic fibrosis is clinically dominated by inflammation and infection [69]. Inflammatory stimuli control CFTR expression at both transcript and protein levels [70–72]. CFTR is subject to complex and integrated phosphorylation and dephosphorylation, regulated by multiple enzymes including PKA, PKC, Src tyrosine kinase, AMP-dependent kinase, and phosphatases. However, the major function of the CFTR protein appears to be that of a PKA-activated anion channel [73].

After Cl^- , HCO_3^- is the second most important anion transported through CFTR. This was first supported by the observation that pancreatic secretions of cystic fibrosis patients do not contain HCO_3^- [74]. Subsequently, direct HCO_3^- transport by CFTR was documented by several studies in digestive epithelial cells [75]. Similar results were found in the pulmonary tract [76]. In addition, secretions from submucosal cells are particularly acidic in cystic fibrosis [77]. CFTR has a dual relation to HCO_3^- in the airways: On the one hand, HCO_3^- is transported through CFTR and on the other hand its function can be regulated by HCO_3^- via sAC (Fig. 2).

It is well established that CFTR is activated by cAMP [78] and sAC contributes. Indirect evidence for this contention was provided by the observation of bicarbonate-induced phosphorylation of CFTR in corneal epithelial cells [79]. The study suggested that sAC coupling to CFTR

occurs in the airways. This reaction is fairly fast, even in the absence of CA. Exacerbations of severe pulmonary diseases may also be associated with alveolar hypoventilation leading to increased CO₂. Together, these mechanisms indicate that there is increased availability of HCO₃⁻ in the airway lumen during exacerbations of airway diseases.

CFTR allows bidirectional Cl⁻ and HCO₃⁻ flux depending on electrochemical gradients. Different conductance directions for chloride in sweat glands and in the airways exemplify this principle. The discussed possibility of increased luminal bicarbonate content during airway disease exacerbations may be a driving force for its conductance into airway epithelial cells. Submucosal glands can produce 20 meq/l HCO₃⁻ (10, 43), which is probably increased by intraluminal production as mentioned above. Intracellular HCO₃⁻ concentrations are described between 10 and 15 mM in mammals [44]. Based on these concentrations, an influx of HCO₃⁻ from the airways into cells, particularly during disease exacerbations when intracellular HCO₃⁻ concentration is lower and the extracellular concentration is higher can lead to stimulation of sAC to increase CBF, when it is most needed. In case of malfunctioning CFTR, there is a decreased secretion of HCO₃⁻ in the submucosal glands and a decreased influx to ciliated cells, indicating the lack of an important rescue mechanism of mucociliary clearance in patients with cystic fibrosis at baseline and particularly during exacerbations. It has to be emphasized that this model needs further experimental confirmation as the here mentioned concentrations of HCO₃⁻ are estimated, but not measured, which is also true for the apical membrane potential of the ciliated epithelial cells. Additionally, the observed effects may be related to altered regulation of other membrane transporters, not only malfunctioning CFTR.

5.1.5. Possible function of estrogens in airways

It is well established that females with cystic fibrosis have a poorer prognosis than males [85–87]. 17β-estradiol (E2) has been shown to increase ENaC activity in alveolar cells of healthy donors [88] and bronchial epithelial cells of women with cystic fibrosis [89]. It has also been shown that the amiloride sensitive nasal potential in women with CF was higher during the luteal phase compared to the follicular phase [90], although we did not find different currents in postmenopausal women compared to females in the reproductive age (unpublished data). Tarran also showed recently that estrogen decreases UTP dependent Ca²⁺ regulation of ASL height [91].

As we have described above, sAC is involved in the regulation of CFTR and CFTR is an important regulator of ASL volume. Catechol estrogens inhibit sAC at an IC₅₀ of 1 μM. The IC₅₀ of tmAC is about 100 times higher (100 μM) [47]. Cytochrome P450 1B1 produces catechol-estrogens by hydroxylation of E2 at a 2' (2-hydroxy 17β-estradiol) or 4' (4-hydroxy 17β-estradiol) position. Interestingly, microarray data from airway epithelial cells from cystic fibrosis patients show a significant expression of cytochrome P450 1B1 (GDS4252), indicating a possible increased concentration of catechol estrogen in the airway epithelium of women with CF. These findings allow hypothesizing a possible role of the described worse clinical presentation of women with CF by inhibition of sAC via increased levels of catechol estrogens.

5.1.6. Role of sAC in the pulmonary endothelial barrier function

sAC as well as bicarbonate transporters have been described in the arterial and venous pulmonary circulation, but only in the venous endothelium a bicarbonate cAMP response was found [92]. Interestingly, cAMP produced by tmAC in the endothelium leads to tightening of the endothelial barrier, whereas sAC activation leads to a weakening of the junctions [92]. This is a clinically important finding as it suggests to not use bicarbonate infusions in patients undergoing permissive hypercarbia in the context of low volume, lung protective ventilation [93] as this may worsen the lung damage in ARDS.

5.2. Role of sAC in other organs

5.2.1. Testis and sperm

sAC isoforms enriched in the testis are involved in sperm motility [94,95] and maturation [95]. sAC mRNA in testes is localized in cell types of late stages in the spermatogenesis, i.e., from round spermatids to early elongated spermatids [96]. The high observed expression levels may indicate an important role for sAC in generating cAMP in spermatozoa required for either sperm maturation, initiation of motility, and/or the acrosome reaction. Moreover, it has been suggested that sAC is produced as a high-molecular-weight precursor protein that is converted to the active form by proteolytic cleavage as the sperm cells proceed through the epididymal tract [10,11]. It was therefore postulated that the activation of sAC results in an increase of the intracellular concentration of cAMP and the induction of the signaling cascade, leading to completion of sperm maturation. Alternative splicing of sAC mRNA may be an additional mechanism responsible for the production of the shorter, active isoform of the enzyme [23].

Interestingly, targeted disruption of a number of genes [97–99], either suggested or shown to be involved in the cAMP-dependent pathway leading to sperm maturation, resulted in fertility defects that were mainly due to impaired sperm motility.

5.2.2. Pancreas

There are proposed roles for sAC in exocrine and endocrine pancreatic function. β-cells release insulin in response to various stimuli including hormones, neurotransmitters and blood glucose levels. It was known for decades that an increase in external glucose concentration stimulates cAMP production while modulating the release of insulin [100] but the source of this cAMP remained unknown until recently [101] when a study using INS-1E insulinoma cells showed that glucose and GLP-1 produce cAMP with distinct kinetics via different adenylyl cyclases. GLP-1 induces a rapid cAMP signal mediated by G protein-responsive tmACs. In contrast, glucose leads to a delayed cAMP rise mediated by HCO₃⁻, Ca²⁺, and ATP-sensitive sAC. These results demonstrate that sAC- and tmAC-generated cAMP define distinct signaling cascades in glucose metabolism [102].

5.2.3. Kidney

Several studies suggested the presence of various sAC splice variants in the kidney [11,24,103,104]. Immunohistochemistry revealed that sAC (or at least a subset of sAC variants) is preferentially expressed in cells of the medullary and cortical thick ascending loop of Henle (TAL), in cells of the distal tubule (DT) and in cells of the collecting duct (CD) [103,104]. Because sAC is present throughout the nephron, it is a good candidate to integrate external (tubular fluid) and internal (plasma, renal interstitium) signals with appropriate responses through cAMP signaling.

Using epididymal proton-secreting cells as a model system, another group identified sAC as a sensor that detects luminal bicarbonate and activates the vacuolar proton-pumping ATPase (V-ATPase) via cAMP to regulate tubular pH. Renal intercalated cells express sAC and respond to cAMP by increasing proton secretion, supporting the hypothesis that sAC could function as a luminal sensor in renal tubules to regulate acid-base balance [105].

Furthermore, sAC is involved in blood pressure homeostasis since specific sAC inhibition by KH7 results in reduced Na⁺ reabsorption [103]. Epithelial sodium channel (ENaC), Na⁺/K⁺-ATPase, and mineralocorticoid receptor (MR) expression may involve binding of phosphorylated CREB-p to CRE at the promoter level [106–108]. A recent study showed that inhibition of sAC by KH7 significantly reduced CREB-mediated promoter activity. Furthermore, KH7 and anti-sAC siRNA significantly decreased mRNA and protein levels of the α subunits of ENaC and Na⁺/K⁺-ATPase. Using atomic force microscopy, significant endothelial cell softening was seen after sAC inhibition. This suggests that sAC is a regulator of gene expression involved in aldosterone signaling and an important regulator of endothelial stiffness [109].

5.2.4. Nervous system

5.2.4.1. Regulation of breathing. The major regulators of acid base are the kidney and the respiratory system. While the kidney is responsible for excreting accumulated acid and retaining HCO_3^- , the respiratory system serves as a fast adjustable switch of CO_2 . While regulation by the kidney takes place in the organ itself, the ventilatory part of the respiratory function is regulated by chemoreceptors outside the lung, namely in the carotid and aortic bodies and the brain stem.

The major determinant of the respiratory ventilatory rate is the arterial partial pressure of CO_2 . Until recently it was felt that the peripheral chemoreceptors were the major O_2 sensor while central chemoreceptors contributed predominantly to the CO_2/pH ventilatory response [110]. Recent evidence suggests that central and peripheral chemoreceptors interact with each other and that the peripheral sensors can modulate the central sensitivity to CO_2/pH [111], recognizing the importance of a functional carotid body for the full response to hypercapnia. In this context, it is interesting that there is a correlation between central sleep apnea and asymptomatic carotid stenosis [112].

There has been a long debate whether it is pH or CO_2 that drives ventilation. sAC investigators got interested in this when elevations in pCO_2 were reported to increase cAMP in glomus cells of the carotid body [113]. sAC expression was found in the carotid body and related non-chemosensitive structures [114]. Transduction of the hypercapnic stimulus into a functional chemoafferent neural signal involves many of the same processes associated with hypoxia sensing. These include type I cell depolarization, Ca^{2+} influx and neurosecretion [115]. However, identification of the specific CO_2 sensing mechanisms in type I cells in response to hypercapnia, remains incompletely defined. Until recently it was also unknown whether CO_2 sensing is mediated by $\text{CO}_2/\text{HCO}_3^-$, decreases in pH or both [110]. In 2013, Nunes et al showed that sAC does not have a physiological role in the cAMP production in isohydric hypercapnia in the carotid body because: sAC expression is lower than tmAC expression, changes in cAMP are not dependent on different HCO_3^- and CO_2 concentrations and not influenced by KH7. No PKA activity could be registered upon different concentrations of HCO_3^- [110]. These findings suggest that the enhanced cAMP generation and carotid body chemoafferent discharge frequency associated with an increase in CO_2 is not mediated directly by $\text{CO}_2/\text{HCO}_3^-$, but is possibly a consequence of a concurrent elevation in intra- and extracellular H^+ generation [110].

5.2.4.2. Other CNS functions of sAC. In the choroid plexus, CO_2 metabolism has long been linked to cerebrospinal fluid (CSF) secretion [116]. sAC mRNA [117], protein [11] and activity [118] have been demonstrated in the choroid plexus, and it can be hypothesized that HCO_3^- regulation of sAC plays a role in CSF homeostasis. In fact, some splice variants of sAC [119] are involved in a novel mechanism of metabolic coupling between neurons and astrocytes. It was shown that sAC is highly expressed in astrocytes. HCO_3^- activation of this enzyme via the Na-HCO_3^- co-transporter (NBC), by either high $[\text{K}^+]$ or glycolysis, increased intracellular cAMP, which leads to glycogen breakdown and the delivery of lactate to neurons for use as an energy substrate [119].

sAC is also present in developing neurons, where, depending on the origin of the neuron, it is located in cell bodies, dendrites, axons and/or growth cones [120]. The effects of sAC overexpression, namely axonal outgrowth and elaboration of growth cones, resemble morphological changes elicited by the treatment of axons with netrin-1. In cultured dorsal root ganglia and spinal commissural neurons, sAC inhibition, either by KH7, catechol estrogens or siRNA, blocked netrin-1-induced growth cone elaboration and axonal growth [120]. Using pharmacological and siRNA approaches, it was found that sAC activity is required for netrin-1-induced cAMP generation leading to netrin-1-mediated growth cone elaboration and axon outgrowth [120].

5.2.5. Eye

In both the corneal endothelium [121] and ciliary body [122], HCO_3^- stimulates fluid secretion. A role for sAC was first suggested by the observation that HCO_3^- stimulates cAMP production in homogenates from both tissues [118]. Subsequent to its molecular isolation, sAC was confirmed to be present in primary cultures of bovine corneal endothelial cells, and sAC activation increased CFTR dependent secretion of Cl^- , HCO_3^- and/or ATP [123]. Although these studies were performed prior to the availability of sAC-selective inhibitors, all data suggest that cAMP produced by sAC stimulates PKA phosphorylation of apical CFTR, thus increasing apical Cl^- permeability [123,124]. It was also demonstrated that higher HCO_3^- in culture media increased sAC expression in corneal endothelial cells [125].

A role for sAC was also investigated in retinal ganglion cells (RGCs). Retinal cells express HCO_3^- transporters and carbonic anhydrases [126, 127]. Krebs cycle-derived HCO_3^- activates mitochondrial sAC, which is a critical metabolic sensor and modulates oxidative phosphorylation [48,128]. Inhibition of sAC activity in RGCs using 2-CE [103] or anti-sAC shRNA decreased RGC survival, while HCO_3^- [125] increased survival and axon growth in RGCs [129]. Another study showed that relative levels of phosphorylated CREB and phosphorylated Bcl-2 were decreased in corneal endothelial cells treated with 2-CE or sAC siRNA, suggesting that HCO_3^- dependent endogenous sAC activity can mobilize anti-apoptotic signal transduction [130].

5.2.6. Skin

Because cAMP plays a vital role in the proliferation, differentiation, and expression of key proteins in keratinocytes, the expression and localization of sAC were examined in normal and diseased human skin [131]. sAC was upregulated in the nuclei of keratinocytes in certain hyperproliferative skin diseases, including psoriasis and squamous cell carcinoma (SCC) in situ. Interestingly, sAC was lost from the nucleus when a malignant epithelial tumor acquired invasive properties in the dermis. The same group previously demonstrated that nuclear sAC was associated with activated CREB and found this trend to also exist in human psoriasis lesions [132]. These data shed light on the complexity of cAMP signaling in skin diseases and suggest that sAC might represent a key player in the pathogenesis of hyperproliferative skin disorders.

6. Summary

Soluble adenylyl cyclase represents a source of cAMP that can be increased via bicarbonate. Because cAMP is a ubiquitous intracellular signaling messenger, the potential physiological effects subjected to sAC modulation are multiple and they include proteins directly sensitive to cAMP like cyclic nucleotide-gated ion channels, exchange proteins activated by cAMP (Epac), as well as proteins sensitive to Epac signaling and PKA-phosphorylation. Immunolocalization studies reveal that sAC is expressed in many tissues, which respond to bicarbonate or carbon dioxide levels suggesting that sAC may function as a general bicarbonate/ CO_2 sensor throughout the body and thus play a fundamental physiological role.

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